



NAVAL MEDICAL RESEARCH UNIT DAYTON

***IN VITRO* CYTOTOXIC POTENTIAL OF AFGHANISTAN
SAND EXTRACT**

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Abstract

U.S. troops deployed to the Middle East are often exposed to dust storms with particulate matter (PM) levels above the military exposure guideline of $150 \mu\text{g}/\text{m}^3$ which can result in an increased incidence of pulmonary diseases. Millennium Cohort Study results also substantiate these respiratory health concerns. Further, metals and harmful chemical components present in Middle Eastern sand have been identified as contributing factors for toxicity. In order to study the toxicological mechanisms in neuronal cells, we evaluated the effects of the soluble components of Afghanistan sand on rat dopaminergic neuronal cell (MES 23.5). Sterilized sand samples (50% W/V) were extracted overnight in serum free media. Different concentrations of extracted samples from Afghanistan sand (equivalent to 100-500 mg of sand/ml) were added to the neuronal cells for 24 hr and cytotoxicity assessed estimating cellular release of lactate dehydrogenase (LDH) and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. In neuronal cells, exposure to Afghanistan sand dust extract increased LDH release dose-dependently with statistically significant increase at higher concentrations (400 and 500 mg/ml), and decreased MTT metabolism. In addition, sand extract exposure resulted in significant reduction in total antioxidant capacity (TAC) with an elevation of reactive oxygen species (ROS). However, N-acetyl cysteine (NAC) pretreated cells showed decreased sand extract-induced ROS and cytotoxicity. This indicated that Afghanistan sand extracts produced cytotoxicity through an oxidative stress mediated mode of action. Moreover, the toxicity was associated with mitochondrial dysfunction (reduced membrane potential) followed by release of cytochrome c and increased caspase-3 activity. All these apoptotic components activated by sand extracts were significantly reduced in the presence of cyclosporine A, NAC or z-VAD (caspase-3 inhibitor). Taken together, this study indicates that soluble components of Afghanistan sands can be toxic to neuronal cells in culture by enhancing ROS and impairing mitochondrial function, although the relevance of these results to the *in vivo* situation remains unclear.

Keywords: Sand, Particulate Matter, Neurons, Cytotoxicity, Oxidative Stress

TABLE OF CONTENTS

Introduction	6
Materials and Methods	8
Neuronal Cell Culture and Sand Exposure	8
Assessment of Cytotoxicity	9
Total Anti-Oxidant Capacity	10
Measurement of Oxidative Stress	10
Mitochondrial Membrane Potential	11
Caspase 3 Assay	11
Cytochrome c Release Assay	11
Statistical Analysis	12
Results	12
Discussion	14
Conclusion	17
References	24

LIST OF FIGURES

Figure 1. Cytotoxicity of Afghanistan sand Extract.....	19
Figure 2. Oxidative Stress is involved in sand-induced toxicity	20
Figure 3. Sand-induced mitochondrial disturbance.....	21
Figure 4. Cytotoxic effects are mediated by mitochondrial dysfunction	22, 23

TABLE

Table 1. Sand Extracts Elemental Analysis Data.....	18
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Introduction

U.S. military personnel deployed to the Middle East in support of Operation Iraqi Freedom and Operation Enduring Freedom have concerns regarding exposure to elevated levels of dust from blowing desert sand (Englehardt, 2007; Bowman, 2004). Desert dust easily enters the atmosphere as a result of natural (e.g., wind erosion) or anthropogenic (e.g., vehicle/personnel movement, construction activities) processes (McDonald and Caldwell, 2008). Numerous metals (Lyles et al., 2008; Perdue et al., 1992; Weir, 2004) and pathogens (Lyles et al., 2005; Griffin, 2007) have been detected in particulate matter (PM) collected from the Middle East. Airborne PM has been linked to a range of serious respiratory and cardiovascular health problems. It is well recognized that deposition of a variety of PM in the lung can result in the generation of inflammatory cytokines and the subsequent development of lung injury (Dreher et al., 1997; Gavett et al., 1997; Kodavanti et al., 1999; Scapellato and Lotti, 2007). The key health effects associated with exposure to ambient PM include: increased risk of myocardial infarction, premature mortality, decreased lung function and aggravation of asthma, chronic bronchitis, and other respiratory and cardiovascular disease (Gordon, 2007; Simkhovich et al., 2008). Recent epidemiologic studies estimate that exposures to PM among the general US population may result in tens of thousands of excess deaths per year, and many more cases of illness (Simkhovich et al., 2008). Recent studies further suggest that transition metals, endotoxins and other contaminants within particulate matter may actually mediate the adverse effects (Donaldson and MacNee, 2001). An association between metal-containing particulates and increased lower respiratory-tract infections and chronic fibrotic lung disease has been well documented in occupational environments (Howden et al., 1988). Wallenborn et al. (2007) also demonstrated that PM associated metals can produce localized lung inflammation and be translocated to extra pulmonary organs following intra-tracheal instillations.

Inhalation of sand dust has been associated with a variety of adverse health effects. Smith and co-workers (2009) have shown that deployment to Iraq and Afghanistan where sandstorms are common was associated with increased respiratory symptoms in ground-based military personnel. Inhabitants of deserts can also develop Desert Lung Syndrome, a rare non-progressive non-occupational dust pneumoconiosis resulting from silica-containing dust deposition in the lungs (Bar-Ziv and Goldberg, 1974). This syndrome generally develops after years of heavy

exposure to sand particles (Nouh, 1989). An acute desert-related lung disease described as Desert Storm Pneumonitis was found to occur following inhalation of pigeon droppings and fine Saudi dust (Korenyi-Both et al., 1992; 1997). Sporadic cases of severe acute eosinophilic pneumonitis with unknown etiology have also been reported among several U.S. military personnel deployed to Southwest Asia (Shorr, 2004). Asian sand dust exposure is associated with increased daily mortality in Seoul, Korea, and Taipei, Taiwan (Kwon et al., 2002) and cardiovascular and respiratory dysfunction in Taipei (Bell et al., 2008).

Although the historical focus of PM toxicity has been cardiopulmonary targets, it is now appreciated that inhaled nano-size (<100 nm) PM particles quickly exit the lungs and enter the circulation where they can be distributed to various organ systems (i.e., liver, kidneys, testes and lymph nodes) (Kreyling et al., 2002; Oberdörster et al., 2002; Takenaka et al., 2001). Damage to these secondary targets can also occur through oxidative stress pathways (Cordier et al., 2004; Samet et al., 2004; Pohjola et al., 2003). The brain is vulnerable to oxidative stress damage because of its high energy use, low levels of endogenous scavengers (e.g., vitamin C, catalase, superoxide dismutase etc.), high metabolic demands, extensive axonal and dendritic networks, and high cellular content of lipids and proteins (Mattson, 2001). The possibility that the brain might also be affected by PM was first raised in a 2002 editorial (Oberdörster and Utell, 2002) and followed by reports showing that nano-size particles could cross the blood brain barrier (BBB) (Lockman et al., 2004) and physically enter the central nervous system (CNS) of animals in small numbers (Kreyling et al., 2002).

There is also evidence that inhaled particles can reach the brain, either by transport along the olfactory nerve or possibly by penetration of BBB that is compromised by systemic effects of PM (Oberdörster et al., 2002; 2004). The activities of signaling pathways that mediate inflammatory responses can be up-regulated in the brains of mice exposed to concentrated ambient particles (CAPs) derived from areas near primary emission sources (Campbell et al., 2005). In addition, biomarkers of oxidative stress and tissue injury in the brain are observed at a higher concentration in mice following exposure to CAPs for as long as 2-weeks post-exposure (Campbell et al., 2005).

Based on the high exposure levels and the range of contaminants present in the PM, ambient particulates in the Middle East may pose a health hazard. This pilot study was designed to examine the potential cytotoxic effects following exposure to soluble components of Afghanistan sand obtained from military-occupied regions.

Materials and Methods

The MES 23.5 cell line derived from somatic cell fusion of rat embryonic mesencephalic cells and the murine neuroblastomaglioma cell line N18TG2, was obtained from Dr. C. Rochet (Purdue University, West Lafayette, IN). MES 23.5 cells are conditionally-immortalized neurons with dopaminergic characteristics, extensively used for *in vitro* toxicity study (Parikh et al., 1995; Zhang et al., 2009; Prabhakaran et al., 2009). Cells were cultured on poly-l-lysine-pre-coated plates in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum(FBS), 2% new born calf serum (NBS), 15 mM HEPES and SATO components (insulin 5 mg/ml, transferrin 5 mg/ml, pyruvic acid 48.6 mg/ml, putrescine 4 mg/ml, sodium selenite 5 ng/ml, progesterone 6.3 ng/ml) at 37°C in an atmosphere of 5% CO₂ and 95% air. Throughout this study cells were used for experiments 48 hr after plating. Afghanistan sand sample (raw material) used in these studies was provided by Capt M.B. Lyles at The Navy Bureau of Medicine and Surgery (BUMED).

Sand Extract Preparation

The high levels of sand dust generated from blowing desert sand have different sized dust particles (predominantly small particles of less than 10 µM capable of reaching deep lungs) with soluble metal contents that may possibly cause health problems (desert lung syndrome, desert storm pneumonitis, acute eosinophilic pneumonia, etc.). The trace elemental analysis of sand dust extracts through inductively coupled plasma mass spectrometer (ICP-MS) showed the presence of several metals at low level compared to raw sand materials (Table 1). Analysis was performed at Dr. José Centeno Lab, Division of Biophysical Toxicology, Department of Environmental and Infectious Disease Sciences, U.S. Armed Forces Institute of Pathology, Washington, DC. To understand the soluble components of sand effects on neuronal cells, bulk sand samples collected from Afghanistan (Khost) were autoclaved (to eliminate bacterial contamination during cell culture) upon receipt and were extracted. 50% w/v sand extracts were

prepared, 10 g of sand + 20 ml of medium without FBS. The day before exposure samples were kept on an orbital shaker overnight at room temperature. Extracts were filtered through a 0.2 μ m filter and stored at 4°C until cells were exposed. Neuronal cells were exposed to extracts with varying concentrations equivalent to 100-500 mg/ml of sand for 24 hr and cytotoxicity was assessed by lactate dehydrogenase (LDH) and MTT assay. For all other assays (total antioxidant capacity, reactive oxygen species generation, mitochondrial membrane potential, caspase-3 activity and cytochrome c release), neuronal cells were exposed to 500 mg/ml sand extracts only. For control cells, DMEM medium was used.

Assessment of Cytotoxicity

Lactate dehydrogenase assay:

The lactate dehydrogenase assay is used to evaluate cell-membrane integrity because the release of this large (9-160 KD) enzyme from the cytoplasmic compartment to the supernatant of cells is indicative of membrane damage. Based on the reduction of nicotinamide adenine dinucleotide (NAD) by the action of LDH to form a tetrazolium dye, the amount of LDH was measured spectrophotometrically at 492 nm as per the method of Vassault (1983). The background absorbance measured at 660 nm was subtracted from the reading at 492 nm. Cells grown in a 12-well plate at a density of 1×10^6 cells/well in HBSS were exposed to a range of sand extract concentrations (100, 200, 300, 400 and 500 mg/ml) for 24 hr and determined LDH release into the medium.

MTT assay:

The MTT assay is based on a colorimetric system, in which tetrazolium rings of the pale yellow MTT are cleaved to form dark blue formazan crystals by the activity of a mitochondrial dehydrogenase enzyme from viable cells. The number of healthy cells can be quantified by spectrophotometric measurement. The cells were seeded in a 12-well plate at 1×10^6 cells/well in Hank's balanced salt solution (HBSS) and exposed to sand extract concentrations (100, 200, 300, 400 and 500 mg/ml) for 24 hr and MTT assay was performed using a microplate reader as per the procedure (Prabhakaran et al., 2009). MTT reconstituted in phosphate buffered solution (PBS) was added to each well. Following 24hr incubation, formazan crystals were pelleted by

centrifugation and dissolved in a MTT solubilization solution. The absorbance was read at 550 nm minus the background at 660 nm.

Total Antioxidant Capacity Assay

The total antioxidant capacity (TAC), accounting for total hydrophilic scavengers, was assayed in cytosolic fractions of cell lysates by a spectrophotometric method according to manufacturer's protocol (BioVision). Briefly, TAC was quantitated after exposure to sand extracts (500 mg/ml) in the presence and absence of NAC (500 μ M) for 24 hr. At the end of exposure cells were washed three times with PBS, scraped off from the inserts and transferred to Eppendorf tubes. After centrifugation (Eppendorf Mini spin Plus, 850 x g (rcf) 10 min, 6°C), the supernatant was removed, 100 μ l cold buffer was added and the cell solution was stored on ice. Afterwards, the samples were vortexed and sonicated for 2 min on ice. The samples were then centrifuged for a second time at 25,000 x g (rcf) at 6°C for 15 min and supernatant was used for the assay. As standard solutions, different concentrations of Trolox (a water-soluble tocopherol analogue) were used. The test samples were added to the wells. Each sample and standard was measured in duplicate. At the end, hydrogen peroxide was quickly added to stop the reaction. The plate was covered and incubated on a shaker at room temperature for 5 min and read absorbance at 570 nm. The resulting TACs are indicated in Trolox equivalents.

Measurement of ROS generation

MES 23.5 cells grown in a polystyrene 96-well plate at a density of 30,000 cells per well were treated with 500 mg/ml of sand extract for 24hr in the presence and absence of NAC (500 μ M). At the end of 24 hr exposure, generation of reactive oxygen species (ROS) was assessed by an oxidation-sensitive fluorescent probe DCFH-DA as per the method of Gunasekar et al. (1995). DCFH-DA is a non-polar compound that readily defuses into cells, where it is cleaved by intracellular esterases to form DCFH and, thereby, is trapped inside the cells. DCFH is oxidized to the highly fluorescent 2, 7-dichlorofluorescein (DCF) by ROS. Cells were loaded with 30 mM DCFH-DA (Molecular Probes, Eugene, OR) for 30 min at 37°C in the dark and then washed with PBS to remove free DCFH-DA. Following sand extract exposure, the culture medium was removed and cells washed twice with PBS. Fluorescence intensity was monitored at excitation

wave length of 485 nm and emission wavelength of 535 nm. Values were expressed as percent of untreated control groups.

Mitochondrial membrane potential ($\Delta\Psi_m$)

The changes in cellular mitochondrial membrane potential ($\Delta\Psi_m$) due to sand exposure was monitored using rhodamine 123 (R123) as previously described (Prabhakaran et al., 2005). Since the loss of $\Delta\Psi_m$ has been linked to the opening of the high-conductance mitochondrial pore transition (MPT), CsA, a blocker of MPT, was used to preserve mitochondrial function. MES 23.5 cells grown in polystyrene 96-well plate at a density of 30,000 cells per well were treated with 500 mg/ml sand extract for 3hr in the presence and absence of CsA (10 μ M). Cells were washed twice in Krebs–Ringer Buffer and loaded with 10 mM R123 and then incubated further at 37°C for 30 min in the dark. Following incubation, cells were washed again twice with Krebs–Ringer buffer, and changes in R123 fluorescence were monitored using a fluorescence plate reader at 498 nm excitation and 525 nm emission. Uptake of R123 into mitochondria is a direct reflection of its permeability; an increase of R123 fluorescence reflects a lowering of $\Delta\Psi_m$.

Caspase-3 protease activity

The cleavage of the substrate Ac-DEVD-pNA was used to determine caspase-3 protease activity according to the manufacture's protocol (BioVision Inc., Mountain View, CA). Following sand extract exposure (500 mg/ml) in the presence and absence of CsA (10 μ M) or z-VAD (100 μ M) for 12 hr, cells were harvested in PBS and centrifuged at 500 \times g for 5 min. The cell pellet was further lysed on ice with lysis buffer for 10 min and then centrifuged at 30,000 \times g (rcf) for 1 min at 4°C. The supernatant was harvested and 80 μ g total proteins incubated with buffer containing 10 mM dithiothreitol and 5 μ l of Ac-DEVD-pNA (final concentration 200 μ M) at 37°C. The chromophore P-nitroanilide was determined at 405 nm with a micro titer plate reader.

Cytochrome c release assay

Quantitation of cytosolic cytochrome c was determined using a cytochrome c ELISA kit (R&D Systems, Minneapolis, MN) as described previously (Kaul et al., 2003). Briefly, MES cells (5 $\times 10^6$ cells) grown in culture wells in serum-free media were exposed to 500 mg/ml of sand extract for 3 hr in the presence and absence of CsA at 37°C. After exposure, cells were collected,

washed once with ice-cold phosphate-buffered saline (PBS; pH 7.4), and resuspended in 1 ml of ice-cold homogenization buffer (10 mM Tris-HCl pH 7.5, 0.3M sucrose, 1 mM phenylmethylsulfonyl fluoride, 25 mg/ml aprotinin, 10 mg/ml leupeptin). Following homogenization, cells were centrifuged at 15,000 x g (rcf) for 60 min at 4°C. Resulting supernatants were collected as cytoplasmic fraction and used to measure cytochrome c release as per the protocol provided by the manufacturer. Optical density of each well was then measured at 450 nm using a micro plate reader (VERSA MAX).

Statistics

Data were expressed as mean \pm standard error of the mean (S.E.M). One-way analysis of variance (ANOVA) followed by Tukey–Kramer procedure for multiple comparisons were used to determine statistical differences between treatments. Differences were considered significant at $p \leq 0.05$.

Results

In this pilot study, dopaminergic MES 23.5 cells were used in order to study potential mode(s) of sand-induced toxicity. Cytotoxicity was evaluated following 24 hr of sand exposure by estimating LDH and MTT assays. Cell viability decreased with increased concentration of sand extracts as observed through increased LDH leakage and reduced mitochondrial function (MTT assay) (Fig 1 A & B). Thus, exposure to sand extracts for 24 hr resulted in significant cell damage/cell death in a dose-dependent manner above 300 mg/ml. Hence, neuronal cells used for all other assays were exposed to the highest dose (500 mg/ml) that caused 50% of the cell death.

It is well known that oxidative stress generated by the cellular response to external stimuli can be due to either increased free radical production and/or the weakening of the cell antioxidant defenses, including antioxidant enzymes, lipophilic and hydrophilic scavengers. We measured the levels of non-enzymatic hydrophilic total antioxidant capacity and generation of intracellular ROS in cells exposed to sand extracts. ROS was determined by loading cells with DCFH-DA, with the resulting increased fluorescence reflected by direct oxidation of DCFH. Exposure to sand extract significantly reduced the total antioxidant capacity (TAC) (Fig 2A) while increasing the accumulation of ROS (Fig 2B). Pretreatment of cells with NAC (GSH precursor) restored

TAC of cells and reduced ROS levels, further confirming the stimulation of intracellular ROS generation (Fig 2A & B) following exposure to sand extracts.

Since mitochondria have been reported to be a primary target of oxidative free radicals (Weitsman et al., 2003), in the present study, sand extracts effect on mitochondrial function was determined. A 3hr treatment with sand extracts (equivalent to 500 mg of sand /ml) reduced the $\Delta\Psi_m$ (increased fluorescence) compared to control cells (Fig 3A). This was further supported by mitotracker red uptake study which showed reduced mitochondrial live cells in sand exposed neuronal cells (Fig 3B). Presence of CsA blocked the sand extract-induced reduction in $\Delta\Psi_m$, supporting the involvement of MPT in the mitochondrial dysfunction.

The loss of mitochondrial membrane potential and opening of MPT have been linked to the release of cytochrome c from mitochondria. To determine whether exposure to sand extracts can affect mitochondria and thereby release cytochrome c, the cytosolic fraction collected from exposed cells were used to determine the levels of cytochrome c by ELISA. As shown in Fig 4A, a 3 hr treatment with 500 mg/ml sand extracts released cytochrome c into the cytosol. However, blocking MPT with CsA preserved the association of cytochrome c with the mitochondria. Further, to determine if the loss of cell viability was caspase-dependent, caspase activity was measured following sand extracts exposure. We observed increased caspase-3 activity following sand exposure in neuronal cells, while zVAD-fmk (a broad spectrum caspase inhibitor) or CsA pretreatment significantly reduced the activity (Fig 4B). This suggests that an apoptotic mode of cell death is initiated in neuronal cells following exposure to the sand extracts. To conclusively demonstrate the involvement of oxidative stress in sand dust-induced mitochondrial mediated cell death, the effects of antioxidant (NAC) and the MPT blocker (CsA) on cell viability were examined. As shown in Fig 4C, both NAC and CsA pretreatments significantly reduced sand extract exposure-induced loss of viability. These results suggest that exposure to sand extracts rapidly increases cellular oxidative stress accompanied by loss of $\Delta\Psi_m$ and caspase-3 mediated cell death.

Detection of Soluble metal constituents

In the present study, the elemental analysis of Afghanistan sand dust extract through ICP-MS revealed a number of metals existence including barium, strontium, copper, arsenic, nickel, iron,

potassium, manganese, and chromium (Table 1). Additional heavy metals (aluminum, titanium, zinc, lead, cadmium, cerium, and lanthanum) were measured but the levels were at low detection limit.

Discussion

There are currently thousands of U.S. military personnel deployed to Afghanistan who confront uncertain environmental and geological health hazards. One issue that has long been posed to military men and women is that of inhalation of PM. Massive sand storms are common throughout the Middle East, and soldiers deployed to these areas are associated with induction of respiratory complaints, in addition to dermal and ocular conditions. The report indicates that the majority of the particulates in sand collected from a site in Afghanistan have elevated levels of PM_{2.5} which are health concern (Engelbrecht et al., 2009). These particles, when inhaled, can be deposited deep into the lungs. Thus, if potentially toxic agents are bioavailable and diffuse into the blood, they then could be carried throughout the body potentially affecting the central nervous, endocrine and immune systems, among other target tissues and organs. Based on the high exposure levels and the range of contaminants present in the PM, ambient particulates in the Middle East may pose a health hazard.

In the present study, we observed that sand derived PM extracts significantly induced neuronal cell death by a mechanism involving the generation of free radicals and activation of the mitochondria-regulated death pathway. Among a variety of environmental pollutants or bioorganic materials adsorbed onto Afghanistan dust particles, we evaluated how soluble components of sand dust material contribute to cell damage. In this study the cytotoxic effects on the neuronal cells due to the sand extracts were observed with higher levels of sand extracts (>300 mg/ml). A role for the mitochondria-regulated death pathway is suggested through our finding that the sand extracts caused decreases in mitochondrial membrane potential which was accompanied by cytochrome c release and caspase-3 activation. We also observed that presence of an MPT pore blocker CsA and caspase inhibitor (z-VAD) inhibited mitochondrial membrane potential and apoptosis. To the best of our knowledge, there has been no report studying the effect of sand dust on neuronal cell death, particularly using *in vitro* models. This study is the

first to show the effect of soluble sand extract components on a cell death pathway in dopaminergic neurons. Further, MES 23.5 cell lines that were used in this study are excellent model to understand the mechanism of neurotoxicity of any chemicals or PM exposure. Neuronal cells are a major component of the brain responsible for communication, maintenance and functioning of the body and also a potential target for oxidative stress in several disorders (Mattson, 2000; 2001; Yen et al., 2011; Tobon et al., 2010). It was reported earlier that PM particles carry numerous bio-contaminants that are capable of triggering free radical production and cytokine release responsible for mitochondrial membrane damage, inflammation and apoptosis (Øvrevik et al., 2009; Davies and Holgate, 2002). In the present study, the depolarization of mitochondrial membrane potential and free radical generation marks the beginning of apoptosis. A significant reduction of $\Delta\Psi_m$ and caspase-3 activation was observed in NAC treated neuronal cells following exposure to sand extract. Thus, the known oxidative stress property of PM particles and biochemical evidence from our study indicates oxidative stress damage to the neuronal cells.

The role for metal components in the PM dust in neurotoxicity remains unclear. There is growing evidence that the soluble metal component of atmospheric dusts may be responsible for dust-induced pulmonary injury (Chen and Lippmann, 2009). For example, intratracheal instillation of the water soluble component of residual oil fly ash (ROFA) can result in pulmonary inflammation (Dreher et al., 1997), and lung responses vary depending upon the metal content of the ROFA sample (Dreher et al., 1997; Gavett et al., 1997; Kodavanti et al., 1999). Pulmonary toxicity has also been reported with Canadian dusts found in ambient air samples (Adamson et al., 2003; Prieditis and Adamson, 2002). In the present study, a number of metals including barium, strontium, copper, arsenic, iron, potassium, nickel, manganese, and chromium were detected in the soluble extracts of Afghanistan sand sample (Table 1). Similar analytical chemical results have been reported elsewhere (Perdue et al., 1992). Some of these metals might have affected the mitochondrial function through permeabilization of the mitochondrial membrane as reported previously (Belyaeva, 2010; Belyaeva et al., 2012). Thus, the induction of toxicity in neuronal cells in the present study may indicate the possible involvement of metal contribution.

Ambient and occupational exposure to PM has been linked with increased hospitalization and mortality from cardiovascular disease, respiratory disease, and lung cancer (Brook et al., 2004; Vineis and Husgafvel-Pursiainen, 2005). *In-vitro* and *in-vivo* studies have identified the generation of reactive oxygen species (ROS) and increased oxidative stress as the primary biological processes that may contribute to such a variety of diverse health effects (Li et al., 2003; Chahine et al., 2007). Epidemiologic (Brook et al., 2004; Schulz et al., 2005) and *in-vivo* studies (Chen and Hwang, 2005) suggest that both PM mass and PM metal components contribute to ROS generation. The brain is vulnerable to oxidative stress damage because of its high energy use, low levels of endogenous scavengers (e.g., vitamin C, catalase, superoxide dismutase), high metabolic demands, extensive axonal and dendritic networks, and high cellular content of lipids and proteins (Mattson, 2001). The possibility that the brain might also be targeted by PM was first raised in a 2002 editorial (Oberdörster and Utell, 2002) and followed by reports showing that nano size particles could cross the blood-brain-barrier (Lockman et al., 2004) and physically enter the central nervous system (CNS) of animals in small concentrations (Kreyling et al., 2002). Beyond this mechanism, the brain may also be targeted through the olfactory pathway while exposed to some of the metals that exists in sand extracts *via* inhalation (Tjalve and Henriksson, 1999; Brenneman, et al., 2000; Persson et al., 2003).

Studies exploring the pathogenic mechanisms of PM have implicated a role for ROS derived from metal-catalyzed reactions (Knaapen et al., 2002; Soukup et al., 2000; Donaldson and Brown 1997). The soluble metal component in PM is also implicated in generating ROS, such as hydroxyl radicals, and causing the release of inflammatory cytokines such as IL-8 and IL-6 from cultured respiratory epithelial cells (Donaldson and Brown 1997, Dellinger et al., 2001). Collectively, these findings suggest that metal-derived free radicals mediate PM-induced DNA damage and apoptosis of cultured respiratory epithelial cells.

Using a mechanistic approach in the present pilot study, we showed that Afghanistan sand extract contents produced toxicity in neuronal cells. While this is the first study to specifically investigate the neuronal effects of Afghanistan sand extracts, data are available on adverse health effects of Middle East dust. A study by Struve et al. (2010) used particles from different regions of the Middle East and founded respiratory cellular toxicity in pulmonary epithelial cell culture model. Increased LDH and decreased MTT following 24 hr exposure to metals and PM samples,

leading to cytotoxicity and this was increased with increased duration of exposure. In the present study, the room temperature extractions of sand dust for 24 hr produce concentrations of materials in the extractant that may not mimic the concentrations that might be expected in the brain following inhalation of PM in a real world setting. *In vivo* studies are required to confirm the relevance of the *in vitro* work using sand extracts. Although the relevance of our *in vitro* findings to *in vivo* conditions requires further in-depth study, there is some supportive information showing that PM induces adverse consequences after short-term exposure periods. In addition to Struve et al (2010) report, studies by Gurgueira and associates (2002) indicated that rats exposed to aerosolized PM for 3-5 hr showed increase in ROS, as assessed by chemiluminescence, and increase in the activities of adaptive antioxidant enzymes (e.g., catalase and superoxide dismutase). Salvi and coworkers (1999) demonstrated that a 1-hr exposure of normal individuals to diesel exhaust increased acute inflammatory responses in the airways. Gilmour and associates (1998) demonstrated that toxic particulates rapidly cross the epithelium and induce interstitial inflammation by a mechanism involving ROS.

Conclusion:

In summary, our study indicates that *in vitro* exposure of rodent neuronal cells to the soluble extract of Afghanistan sand induces apoptosis *via* a mode of action involving the generation of free radicals and activation of the mitochondria-regulated death pathway. Further studies are needed to elucidate the mode of action of Afghanistan sand dust extract-induced apoptosis and the linkage between mitochondrial mediated cell death and metal contribution in toxicity. Hence, metal chelation and its effects on toxicity may warrant study.

Acknowledgements

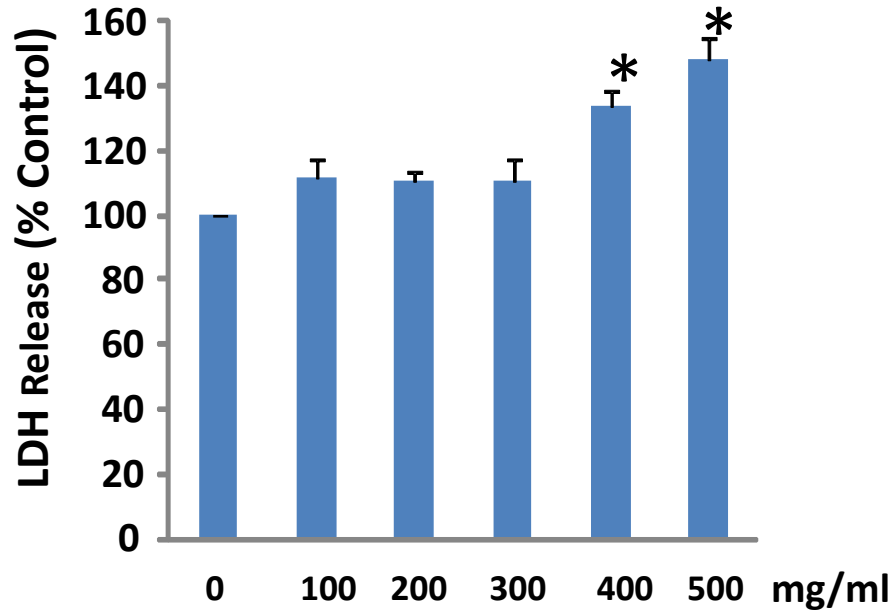
The authors would like to express special thanks to CDR Gail Chapman and LCDR Michael Stockelman for their helpful suggestions and approval of this pilot study.

Table 1: Sand Extract Elemental Analysis Data

	Plain Media	Sand Extract	Raw Sand
Co59(LR)	60	162	20023
Ni60(LR)	<LOD	632	142015
Cd111(LR)	<LOD	<LOD	84
Sn118(LR)	7	<LOD	<LOQ
Sb121(LR)	<LOD	<LOQ	<LOD
W182(LR)	40	<LOQ	<LOD
Ti205(LR)	<LOD	<LOD	203
Pb208(LR)	<LOD	<LOD	11889
U238(LR)	0	5	267
Ba138(LR)	<LOD	383	108063
Ce140(LR)	2	<LOD	20080
La139(LR)	1	<LOD	9117
Li7(LR)	<LOD	<LOD	38186
Sr88(LR)	48	3008	280040
Al27(MR)	2908	<LOD	45373340
Ti49(MR)	25	<LOQ	622322
V51(MR)	<LOQ	<LOQ	86969
Cr52(MR)	23	37	223616
Mn55(MR)	<LOQ	1386	843391
Fe56(MR)	<LOQ	1203	45310882
Fe57(MR)	<LOQ	1242	46353387
Cu65(MR)	97	22	32737
Zn66(MR)	28	<LOD	80884
Mo98(MR)	<LOD	9	156
Sn118(MR)	7	<LOD	<LOQ
Ba138(MR)	<LOQ	397	112365
Ce140(MR)	2	<LOD	21973
La139(MR)	1	<LOD	9679
As75(HR)	0	12	5843
	µg/kg	µg/kg	µg/kg

FIGURE 1

A



B

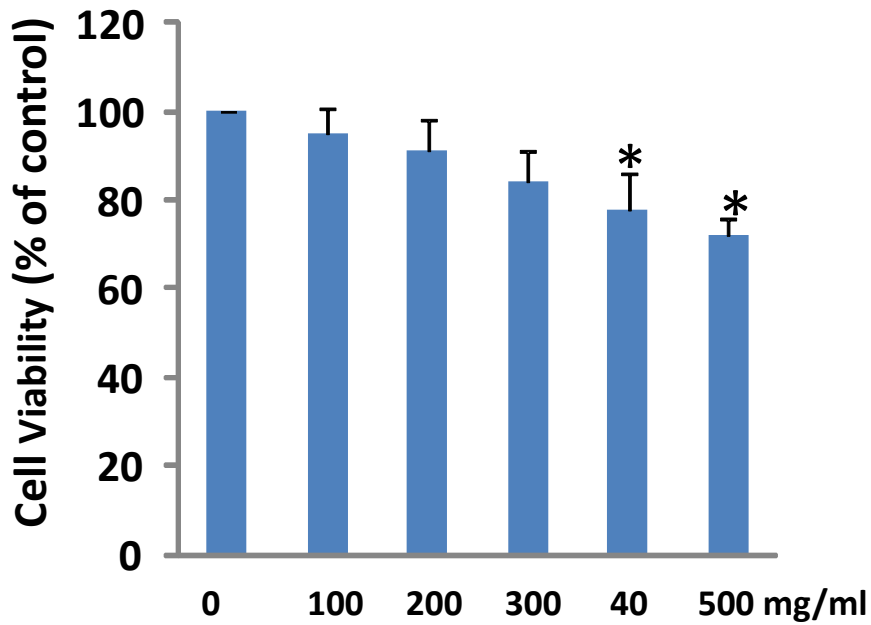
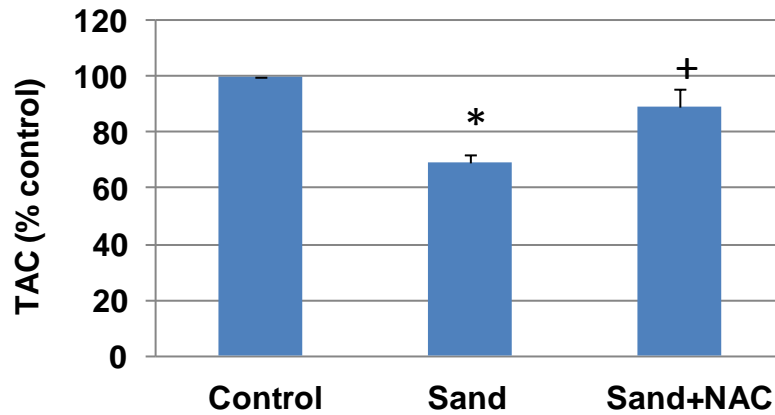


Figure 1: Cytotoxicity of Afghanistan sand extract. Cells were exposed to different concentrations of extract for 24 hr and cell viability assessed by A) LDH release and B) MTT

assay. * Statistically significant effects were found for the extract from 400 and 500 mg of sand/ml at $p \leq 0.05$. Data are the means \pm SEM of 4 experiments for each treatment group.

FIGURE 2

A



B

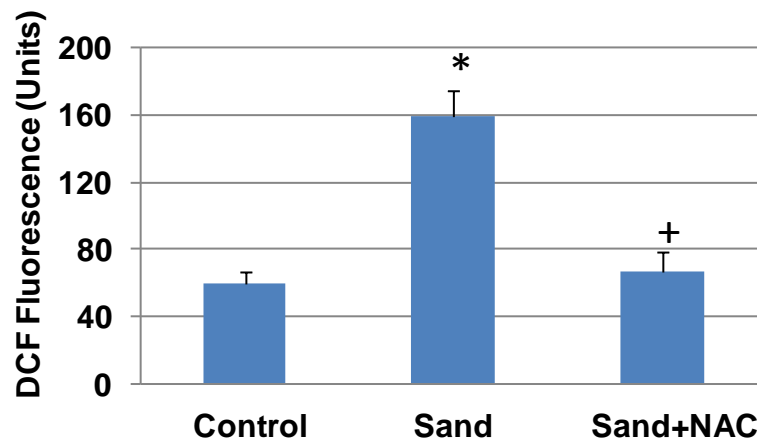
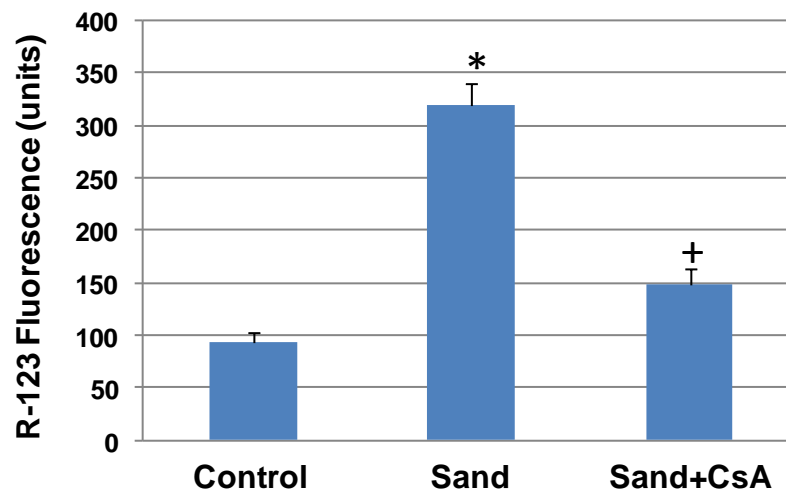


Figure 2: Oxidative stress is involved in sand extract-induced toxicity. A) Total antioxidant capacity after exposure to sand extract (500 mg/ml) with and without NAC for 24 hr. B) Reactive oxygen species generation as measured by DCF fluorescence. Data are the means \pm SEM of 4 experiments for each treatment group. * indicates a statistically significant effect of sand extract vs control and + indicates statistically significant effect of antioxidant NAC vs sand at $p \leq 0.05$

FIGURE 3

A



B

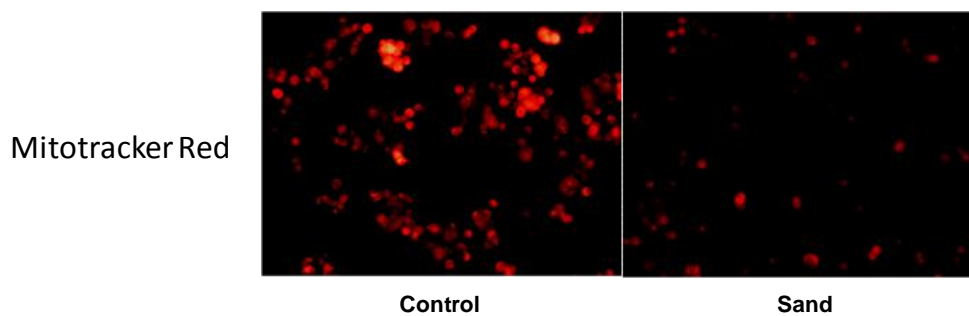
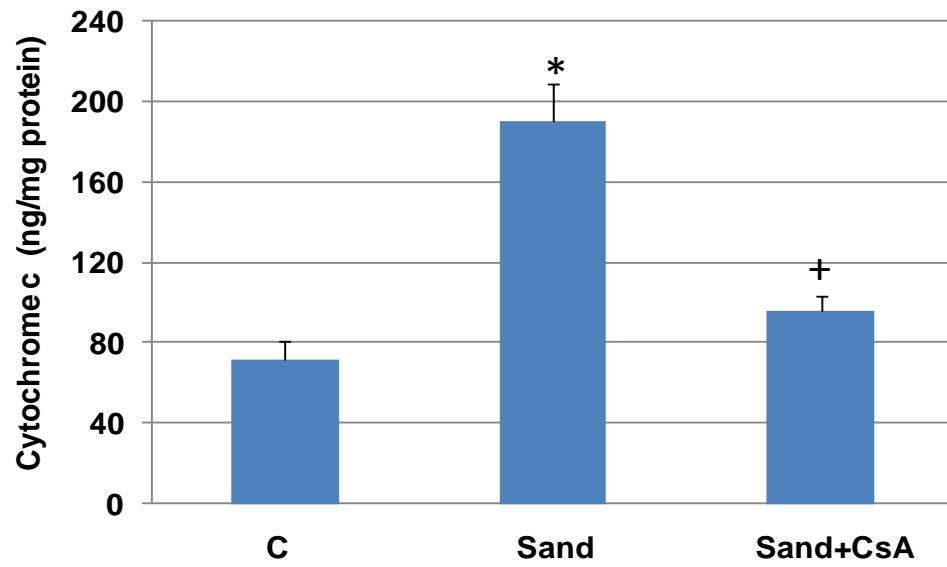


Figure 3: Sand extract-induced mitochondrial disturbance. Cells were treated with sand extract (500 mg/ml) in the presence and absence of CsA for 3hr. A) Mitochondrial membrane potential monitored by Rhodamine-123 Fluorescence. B) Fluorescence photomicrograph using mitotracker red. Data are the means \pm SEM of 4 experiments for each treatment group.

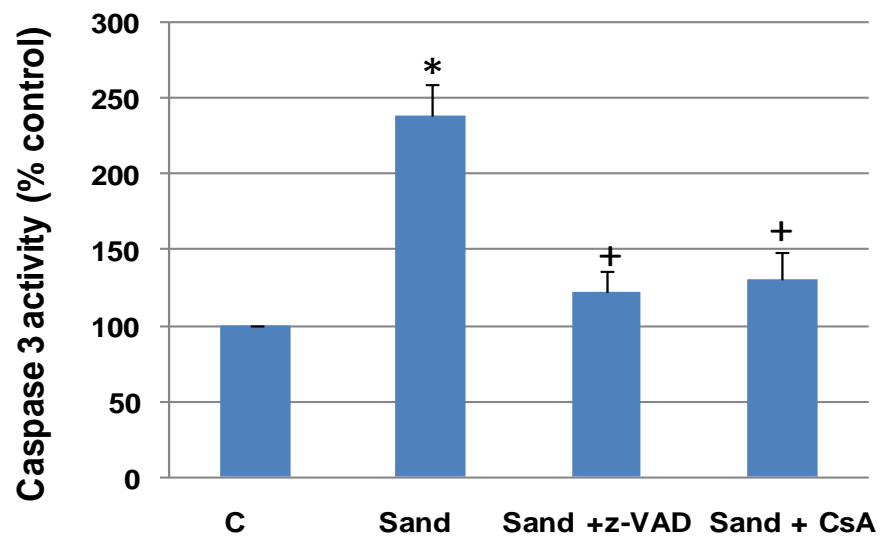
* indicates a statistically significant reduction in membrane potential of cells vs. control cells and + indicates statistically significant effect of the MPT blocker CsA vs sand extract at $p \leq 0.05$.

FIGURE 4

A



B



C

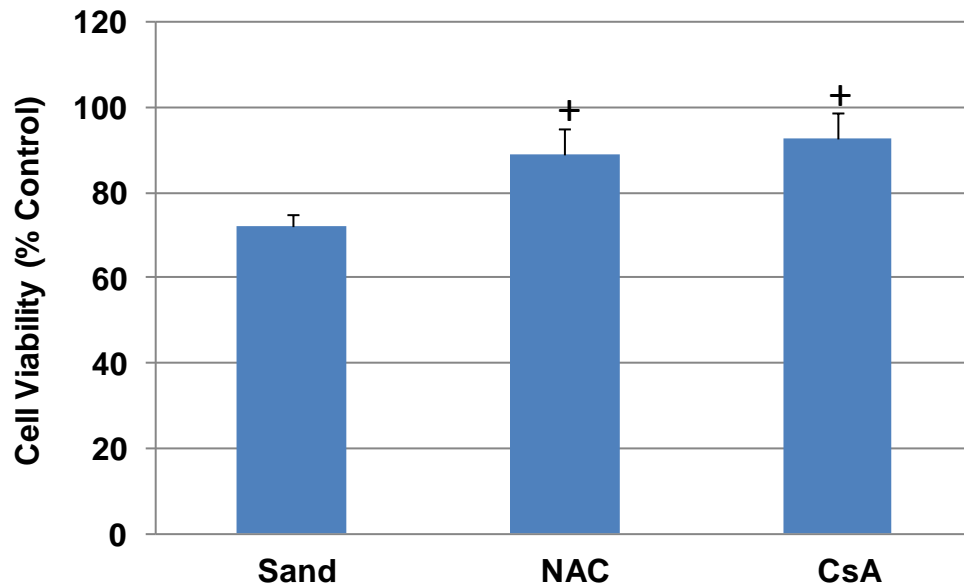


Figure 4: Cytotoxic effects of Afghanistan sand extract is mediated by mitochondrial dysfunction. A) Cytosolic cytochrome c was determined in cells treated with sand extract (500 mg/ml) for 3hr in the presence and absence of CsA. B) Caspase-3 activity was assayed after 12 hr of exposure using sand extract (500 mg/ml) in the presence and absence of z-VAD or CsA. C) Cell viability was determined after 24 hr of exposure to sand extract (500 mg/ml) in the presence and absence of NAC or CsA. Data are the means \pm SEM of 4 experiments for each treatment group.* Indicates statistically significant from control and ⁺ indicate statistically significant from sand extract exposure at $p \leq 0.05$

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